A histochemical double staining protocol for GABA-α2 and CAMKII in hippocampal neurons using PGC-1α transgenic mice as an example

Maria Elisabet Koivisto, PhD

Minerva Institute, Department of Biochemistry and Developmental Biology

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maria.koivisto@helsinki.fi

Instructor: Professor Dan Lindholm

University of Helsinki

Faculty of Medicine

HELSINGIN YLIOPISTO - HELSINGFORS UNIVERSITET

Tiedekunta/Osasto - Fakultet/Sektion – Faculty
Faculty of Medicine

Laitos - Institution – Department
Minerva Institute, Department of Biochemistry and Developmental Biology

Tekijä - Författare – Author
Maria Elisabet Koivisto

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Tiivistelmä - Referat – Abstract
Immunohistochemistry (IHC) is a widely used research tool for detecting antigens and can be used in medical and biochemical research. The co-localization of two separate proteins is sometimes crucial for analysis, requiring a double staining. This comes with a number of challenges since staining results depend on the pre-treatment of samples, host-species where the antibody was raised and spectral differentiation of the two proteins. In this study, the proteins GABAR-α2 and CAMKII were stained simultaneously to study the expression of the GABA receptor in hippocampal pyramidal cells. This was performed in PGC-1α transgenic mice, possibly expressing GABAR-α2 excessively compared to wildtype mice. Staining optimization was performed regarding primary and secondary antibody concentration, section thickness, antigen retrieval and detergent. Double staining was performed successfully and proteins of interest were visualized using a confocal microscope after which image analyses were performed using two different methods: 1) a traditional image analysis based on intensity and density of stained dots and 2) a novel convolutional neural network (CNN) machine learning approach. The traditional image analysis did not detect any differences in the stained brain slices, whereas the CNN model showed an accuracy of 72% in categorizing the images correctly as transgenic/wildtype brain slices. The results from the CNN model imply that GABAR-α2 is expressed differently in PGC-1α transgenic mice, which might impact other factors such as behaviour and learning. This protocol and the novel method of using CNN as an image analysis tool can be of future help when performing IHC analysis on brain neuronal studies.

Avainsanat – Nyckelord – Keywords
Immunohistochemistry, IHC, double-staining, GABA-α2, CAMKII, PGC-1α, machine learning, convolutional neural network, CNN

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Tiedekunta/Osasto - Fakultet/Sektion – Faculty	Laitos - Institution – Department	
Medicinska fakulteten	Minervainstitutet, Avdelningen för biokemi och utvecklingsbiologi	
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Tiivistelmä - Referat – Abstract

Immunohistokemi (IHC) är en metod för undersökning av antigener som används inom medicinsk och biokemisk forskning. Ibland krävs en dubbelfärgning av två separata protein för att klarlägga co-lokalisering. Detta innebär utmaningar eftersom färgningsresultaten beror på ett flertal faktorer, bland annat förbehandling av sampel, värdorganismen där antikropparna odlats och separering av de färgade proteinernas spektra under mikroskop. I denna studie dubbelfärgades proteinen GABAR-α2 och CAMKII simultant för att klarlägga hur dessa protein uttrycks i GABA receptorer i pyramidalceller belägna i hippocampus. Färgningen gjordes hos PGC-1α möss som eventuellt uppvisar en förhöjd expression av GABAR-α2 jämfört med möss av vildtyp. En optimering av dubbelfärgning gjordes beträffande primära och sekundära antikroppskoncentrationer, tjocklek av vävnadssampel, epitop-demaskering och detergenter. De färgade proteinen gjordes därefter synliga med ett konfokalt mikroskop och två olika bildanalyser genomfördes med olika metoder: 1) en traditionell bildanalys baserat på intensiteten och tätheten av färgade områden samt 2) en nyare maskininlärningsmetod baserad på djupa faltningsnätverk (CNN). Den traditionella bildanalysen hittade inga skillnader mellan de transgena mössens och kontrollgruppens vävnadssampel, medan CNN modellen lärde sig att kategorisera bilderna korrekt i respektive grupp (transgen/vildtyp) med en 72% noggrannhet. Resultaten från CNN modellen tyder på att GABAR-α2 uttrycks olika i de transgena mössen jämfört med vildtypen. Detta kan eventuellt påverka betéendemässiga faktorer och inlärning. Detta protokoll samt den beskrivna maskininlärningsmodellen kan vara till hjälp i framtida IHC forskning av neuroner.

Avainsanat – Nyckelord – Keywords

Immunohistokemi, IHC, dubbelfärgning, GABA-α2, CAMKII, PGC-1α, maskininlärning, djupa faltningsnätverk, CNN

Säilytyspaikka – Förvaringställe – Where deposited

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1 INTRODUCTION

Immunohistochemistry (IHC) is a commonly used technique in medical and biochemical research. The technique is based on the binding affinity and specificity of a labeled antibody to its targeted antigen in situ, making it possible to localize and visualize specific cellular components and their histological context (1). Although discovered already in the 1930´s, the use of IHC has shown an exponential growth in the last decades (2).

Simultaneous staining of multiple antigens might sometimes be necessary. For instance, one might be interested in the relative localization of two or more antigens. This gives information about the topographic relations of the antigens, e.g. if the target antigens are present in the same cell populations, in different cells, or even in the same cellular compartment. It can also provide information about cell-cell contacts (3).

Once a cell has been recognized and its proteins mapped, the subsequent step is to connect the cell type with functions. In the brain, this involves linking the cell with information processing and behaviour. One of the brain structures known to have a massive impact on information processing and especially in the formation, organization and retrieval of memories is the hippocampus (4). In this study, we were interested in finding a double staining protocol for GABAR-α2, a ligand-dependent chloride channel mediating inhibition of postsynaptic neurons, and CAMKII, a Serine/Threonine protein kinase functioning as a marker for pyramidal cells, in mouse brain hippocampal neurons. This was done in order to map the expression of GABAR-α2 in pyramidal neurons in an area of the hippocampus known to affect multiple aspects of behaviour. The staining was performed on two strains of mice; mice overexpressing PGC-1α (a peroxisome proliferator-activated receptor γ coactivator 1α), and wildtype mice.

I first describe the general principles of immunohistochemistry and techniques that were applied in this study, along with critical steps for successful immunohistochemical staining. I will then proceed to the significance of the neural circuits in the hippocampus and the role of GABAergic signaling in the brain. Lastly, a novel approach of using machine learning in categorizing IHC stained images will be described.

1.1 Immunohistochemistry as a method

1.1.1 Antibody use in immunohistochemistry

The immune system is our body's way of preventing the outbreak of disease. It can be further divided into innate and adaptive immunity. Innate mechanisms react to pathogens and their components in a nonspecific manner and involve the complement system, macrophages, granulocytes, mast cells and natural killer cells. These cells identify pathogens by the use of pathogen recognition receptors (PPR's) as well as signs of tissue damage, and trigger similar immune responses regardless of the pathogen. The adaptive immunity provides a specific immune response directed at an invading pathogen. It is composed of T- and B-cells that circulate in the bloodstream and lymphatic tissues. As a result of genetic recombination, these cells have a large repertoire of specific antigen receptors that can identify non-self antigens on the surface of cells. When these cells become activated, there is a primary effector response that eliminates or neutralizes the pathogen. They also form memory cells that can induce a more rapid, secondary immune response if they are re-exposed to the same antigen, thus preventing disease (5).

Upon activation, the B-cells produce antibodies or immunoglobulins (hereafter abbreviated Ig) that can bind to specific antigens. There are five classes of antibodies (IgG, IgA, IgD, IgE and IgM), each with specific characteristics and structure (6). The most common class of antibodies is IgG. It is composed of four polypeptide chains: two heavy chains and two light chains, which are linked covalently and with disulfide bonds. Each chain is composed of both constant and varying domains. The constant folds have a structure called the immunoglobulin fold, which is a highly conserved structural motif belonging to the all-β class of proteins. The heavy chains have three constant domains and one varying domain, whereas the light chains have one of each. The varying parts come together, forming the antigen-binding site (Fab). The tail region that interacts with the cell surface receptor is called a fragment crystallizable region (Fc region; Fig. 1). These receptors are commonly found on effector cells of the innate immune response and determine the human subclass of the immunoglobulin (7).

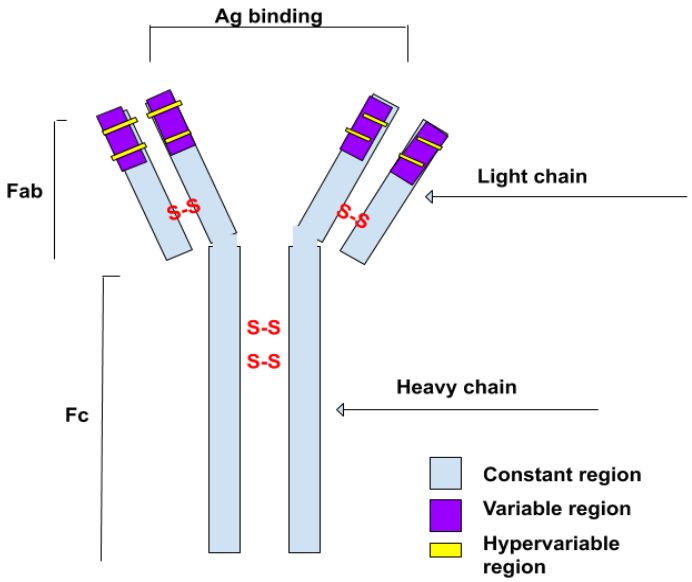


Figure 1. IgG schematic structure.

The binding specificity is determined by the amino acid residues in the variable domain in the heavy and light chains. Specificity is based on complementarity between the antibody and the antigen in regard to shape and location of charged, nonpolar and hydrogen-bonding groups. A match often induces a conformational change, a so-called induced fit (6).

The high binding affinities along with the specificity of antibodies make them useful in analytical approaches. In immunohistochemistry (IHC), antibodies are used to detect the location of proteins and other antigens in tissue sections. Two sorts of antibody preparations can be used: 1) polyclonal antibodies, which are produced by many different B lymphocytes and 2) monoclonal antibodies, which are generated by a single clone of B-cells. The latter are generated by fusing antibody-producing cells with immortalized cell lines, giving rise to a cell line called a hybridoma. Historically, monoclonal antibodies have been produced in mice but in recent years also rabbit monoclonal antibodies are found on the market. Polyclonal antibodies are taken directly from a host animal and affinity purified. They thereby contain a mixture of different antibodies binding to the antigen of interest (8). A major advantage of monoclonal antibodies is that they are consistent in their specificity between different production batches (9).

Antibodies are referred to as primary, secondary or tertiary, the primary antibody being the most critical one since it binds to the target antigen. One can use either a direct approach, where the primary antibody contains a color label, or an indirect approach where a secondary or even a tertiary antibody is used, attaching to the primary or secondary antibody, respectively. The secondary and tertiary antibodies function as signal amplifiers and are conjugated to color labels, which can then be visualized under a microscope. Usually, the color labels contain fluorescent dyes. Both approaches are commonly used, but a disadvantage in the direct approach is that a color label can significantly weaken the binding of the primary antibody. The indirect approach is more common in IHC due to a higher sensitivity level. The use of a secondary antibody is dependent on the species in which the primary antibody was initially generated since it should react against that species (9). For instance, if the primary antibody was raised in a rabbit, the secondary antibody should be anti-rabbit.

1.1.2 Fixation of samples

Fixation refers to the treatment of the collected specimen with one or several chemicals to preserve the tissue and cell structures and to permeabilize them to antibodies and detection reagents. This is a critical step in IHC since improperly prepared tissues will not stain in a desired way (9). Tissue samples must be prepared rapidly in order to avoid degradation processes and autolytic reactions. After collection of tissue, cellular structures containing lysosome rupture and release lysosome due to oxygen deficiency, ultimately leading to cell damage. With the use of fixatives, the lysosomal enzymes are inhibited. Fixatives also precipitate most large molecules in tissue and stabilize cytoskeletal proteins (3).

Fixation can be categorized as cross-linking reactions or precipitating reactions. In cross-linking, one molecule of the fixatives link two molecules of antigens and stabilize them. A widely used fixative in IHC is formaldehyde (CH_2O). Precipitating fixatives cause antigens to precipitate or transform soluble proteins to insoluble aggregates (9). Tissues can also become overfixated, causing the masking of protein antigens and leading to weak non-specific staining and non-specific background staining (3).

1.1.3 Perfusion and handling of tissues

Perfusion refers to a technique where the cardiovascular system is used to replace blood with fixative chemicals, e.g. paraformaldehyde. For small animals like mice or rats, a whole body perfusion can be performed where the fixative is typically injected to the heart, enabling living cells to be fixed in situ quickly. The advantage of this technique is that the fixative can quickly reach all body parts via the body's natural transport system. Smaller pieces of tissue or organs can be immersed directly in fixative chemicals (10).

After fixation, the tissue must be cut into thin sections in order to perform IHC. Thin sections enable the penetration of antibodies and are optically transparent, making it possible to study the cell structure under a microscope (9). The cutting is performed using a microtome, which cuts a tissue sample into thin slices. To be able to do this, the sample needs to be either frozen or paraffin-embedded.

Freezing of tissues is generally easy and does not require any special instruments. Also, the antigens are usually well preserved. However, the durability is often compromised using this technique and samples can be destroyed if a heat-induced antigen retrieval is performed. Paraffin-embedded tissue samples have the advantage of being well preserved even for years and they can be stored at room temperature. However, their preparation usually requires a special instrument and antigens may suffer when exposed to high temperatures during preparation (9).

1.1.4 Blocking and permeabilization of samples

Before staining can be performed, it is necessary to block samples in order to avoid non-specific binding of antibodies. A number of endogenous substances can interfere with staining results, including endogenous peroxidase, endogenous fluorescence, endogenous antibody binding capability (FC receptors), endogenous biotin and unreacted fixative aldehydes (11). Unspecific binding can be prevented by exposing the samples to a solution that contains proteins that will compete for the non-specific binding sites before the antibody is added. Common solutions include bovine serum albumin (BSA), casein, gelatin or normal serum from the host species where the secondary antibody was generated. Low concentrations of a detergent are also used if the sample will be permeabilized to compete for hydrophobic interactions (12).

Permeabilization of the cell membrane (and sometimes nuclear membrane) is an obligatory step in order to detect antigens located inside the cell. Two types of permeabilizing agents are

commonly used: 1) organic solvents like methanol and acetone and 2) chemicals acting as detergents such as saponin, Triton-x and Tween-20. The organic solvents dissolve the lipids from the membrane and coagulate proteins. Saponin interacts with cholesterol in the lipid bilayer, selectively removing it and thereby making small holes in the membrane (13). Triton-x is a non-ionic detergent and contains a hydrophobic group. It is one of the most commonly used detergents in IHC and disrupts the cell membrane. It is harsher than Tween-20, which is considered a better choice if the proteins of interest are located on the cell membrane (11). However, both Triton-x and Tween-20 are non-selective in nature and may therefore extract proteins in addition to lipids (13).

1.1.5 Antigen retrieval

In the fixation process, cross-linked proteins form methylene bridges. This gives rise to net-like structures which can become thick, especially if the fixation time is long. The net can then shield protein antigens, preventing the antibodies from finding them and resulting in poor staining results (9). This problem can be overcome by performing an antigen retrieval.

The two main methods used for antigen retrieval are enzymatic and heat-induced antigen retrieval (=Heat-Induced Epitope Retrieval, HIER) in either low or high pH. Common enzymes used are trypsin, pepsin and proteinase K enzymes. The drawback of both antigen retrieval methods is that the tissue can become physically damaged (9).

The mechanisms for antigen retrieval are not fully understood, but it is assumed that the heat loosens or breaks the cross-linkages from the formaldehyde fixation, improving the penetration of antibodies into the tissues. Some proteins may denature, exposing the epitope (14). Generally, antigen retrieval is recommended for paraffin-embedded sections whereas frozen tissues can be stained without. However, even in unfixed, frozen tissue samples the staining sensitivity has been found to increase significantly when subjected to a HIER (14).

1.1.6 Double staining

Multiple staining comes with a number of challenges: first, most commercial antigens are raised in either mouse or rabbit and visualized with systems based on anti-mouse and anti-rabbit secondary antibodies. If possible, separate host species should be used in order to avoid the secondary antigen from binding to both antigens of interest. Second, spectral differentiation must be clear and colors that are clearly distinguishable should be chosen. Further on, the mixed color should be

in clear contrast with the two basic colors . Third, if targets are not co-localized, it may be difficult to balance signals to enable visualization of the rarer antigen in the same slide as a more commonly expressed target. An adjustment in concentration of the primary antibodies may solve this problem (3). Further on, the two antigens may require different pre-treatments or be visible at differing scales.

There are two basic approaches that can be used when double staining; sequential staining or simultaneous multi-staining. The latter can be used if the antibodies of interest are raised in different species and if the reagents show little or no cross-reactivity. If this is not the case, a sequential approach should be used where one staining procedure follows another.

We chose a simultaneous staining approach since our antibodies were raised in different host species (mouse and rabbit) and since we anticipated both of our antigens to be found in the cytosol. The two antibodies were fluorescence-labeled with fluorochromes emitting different colors to allow direct visualization of both antigens simultaneously. Red and green fluorochromes, commonly used simultaneously in double staining (e.g. 15,16), were used.

1.2 Studying the brain

1.2.1 The glutamatergic and GABAergic system

In order to function, the central nervous system (CNS) requires fast pathways for communication. This is mainly achieved by the synaptic release of two amino acids: glutamate for excitatory synaptic transmission and γ-aminobutyric acid, GABA, for inhibitory transmission. Glutamate is the most important transmitter for brain function and is released from over half of the synapses of the brain. In addition to glutamate and GABA, glycine is another inhibitory neurotransmitter found in the central nervous system. The presence of multiple receptor types enables postsynaptic cells to be both excitatory and inhibitory (17).

GABAergic inhibition is mandatory for upholding the balance of inhibition and excitation and for temporal modulations. Three types of GABA receptors are found; ionotrophic $GABA_A$ and $GABA_C$ receptors and metabotrophic $GABA_B$ receptors, all of which have several undertypes. The ionotrophic receptors are ion channels, mostly letting in chloride and thus inhibiting postsynaptic cells. Many drugs including barbiturates and benzodiazepines can bind to these receptors and cause sedation (17).

1.2.2 Hippocampus – role and cell types

The hippocampus is a bilateral structure found right beneath the neocortex on the basal medial surface of the temporal lobes. It spans all the way from the amygdala to the septa along the temporal lobes. The hippocampus is one of the oldest structures of the brain and it is found in all vertebrates. It receives its main input from the entorhinal cortex and sends out efferent fibers to areas within the hippocampus as well as to other brain structures including the fornix and temporal neocortex. The hippocampus and entorhinal cortex together form an important memory center in the brain. It is part of the limbic system that is active when new memories are formed and also plays a role in learning and emotions (18).

Memories are not stored in the hippocampus long-term. Rather, the hippocampus takes in information, registers it and stores it temporarily. There are several subregions of the hippocampus that are involved in certain types of memories, e.g. the posterior region plays a role in the spatial memory (17).

The hippocampus can be divided into two distinct regions; the dentate gyrus (DG) and the Cornu Ammonis (CA). The DG is V-shaped whereas the CA is a curved structure. The inner part of the DG is known as the hilus. Both DG and CA have a main cellular layer. In the DG it is called the granule cell layer and in CA the pyramidal cell layer. The DG and CA are both composed of several layers. In DG these are the stratum granulosum and stratum moleculare, containing the proximal dendrites of the granule cells. The axons of the granule cells are mossy fibers (MFs) that project to the pyramidal cells of the CA3 region (18).

The CA is composed of seven distinct layers, these being the following in an order from inside out: stratum moleculare, stratum lacunosum, stratum radiatum, stratum lucidum, stratum pyramidale, stratum oriens and the alveus. The CA region can further be divided into four subregions, namely CA1, CA2, CA3 and CA4 (Fig.2) (18).

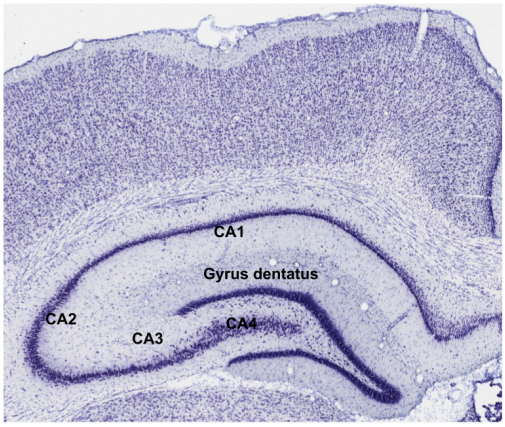


Figure 2. Subregions of the hippocampus.

The connectivity pattern of the hippocampus is arranged in a trisynaptic circuit in the septo-temporal plane. The first node is composed of the pyramidal cells of the entorhinal cortex, which is the main afferent to the hippocampus. The axons of these cells go through the hippocampal fissure to the granule cells. The granule cells then project their MF's to the dendrites of the pyramidal cells of CA3, which is the second link in the circuit. Further on, the pyramidal cells of CA3 then send collateral fibers, so called Schaeffer collaterals, to the pyramidal cells of CA1, constituting the final link in the circuit (Fig. 3) (18).

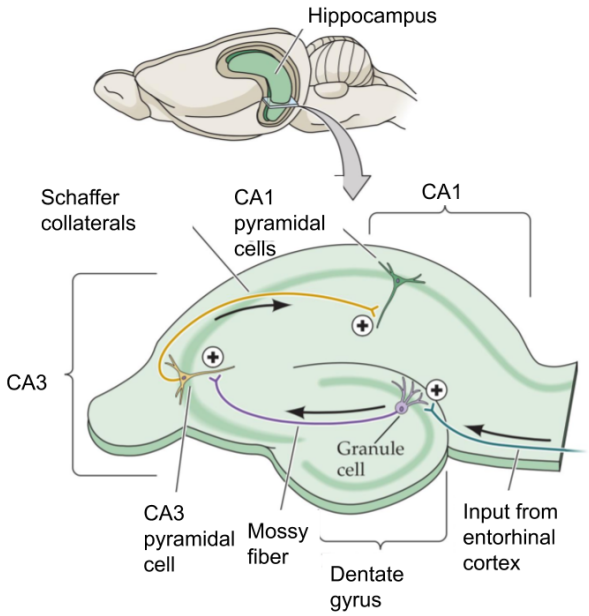


Figure 3. Hippocampal trisynaptic circuit. Picture modified from Purves, Augustine et al. 2012 (17).

The CA1 area of the hippocampus is one of the most studied areas in the brain cortex. It is composed of excitatory pyramidal cells involved in spatial and episodic memories. It also provides glutamatergic, excitatory output to other cortical and subcortical areas. In addition to the glutamatergic cells, the pyramidal cells are surrounded by GABAergic interneurons with an inhibitory function (19).

The synaptic connections between the Schaffer collaterals and CA1 pyramidal cells are involved in long-term potentiation (LTP), a long-lasting increase in synaptic activity involved in memory formation (17). The input comes mainly from the hippocampal CA3 area, local axon collaterals and the amygdala. The dendrites also receive local GABAergic input from interneurons. One CA1 pyramidal cell receives roughly 30 000 synaptic inputs from surrounding cells. The cell body then integrates the input and receives only GABAergic synapses. The axon-initial segment receives only GABAergic synapses (19).

1.2.3 The role of GABA-α2

The $GABA_A$ receptors are composed of pentameric combinations of homologous subunits. There are at least 19 genes encoding $GABA_A$ receptor subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, ρ1–3) with the α-subunits showing the largest diversity (20). About 60 % of the $GABA_A$ receptors have the α1 subunit, 15–20% the α2 subunit, 10–15% the α3 subunit, and 5% the α5 subunit (21). The subunits differ in their expression pattern in the CNS and may be involved with different behavioral functions (20).

The GABA system is tightly linked to anxiety and anxiety disorders as well as anxiolytic drugs, which have been studied extensively in mouse models. It has been shown that α2-containing $GABA_A$ receptors are required for the anxiolytic effects of both diazepam and chlordiazepoxide in anxiety related tests. GABA-α2 has also been linked to alcoholism, depression and the brain reward system in humans (20 and references within). Further on, GABA-α2 is hypothesized to affect the crosstalk between interneurons and pyramidal neurons and disturbances have been linked to disorders such as schizophrenia (20,22,23).

In the hippocampus, $GABA_A$ receptors modulate mossy fiber excitability and alter Ca^{2+} influx into axons. The GABA-α2 protein is present in high numbers in mossy fiber terminals in the stratum lucidum of CA3, the termination region of mossy fibers (24). It is also found in the CA1 region pyramidal cells that receive a high amount of GABAergic input (25).

1.2.4 The role of CAMKII

CAMKII plays a central role in the coordination and execution of the Ca^{2+} signal transduction (26). Like most other CAMKs, CAMKII is a Serine/Threonine protein kinase and it is the most common postsynaptic protein at Schaffer collateral synapses. CAMK is involved in the induction of long-term potentiation (LTP). Calcium influx via NMDA-receptors activates CAMK, resulting in the phosphorylation of AMPA receptors. This enhances the response to glutamate in postsynaptic neurons (17). CAMKII is able to autophosphorylate, making it a Ca^{2+}-independent enzyme. This autonomy is required for memory formation in the hippocampus (27). In this study, CAMKII was used as a marker for pyramidal neurons (15,28).

1.2.5 PGC-1 α TG mice as an example

The peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) is an important regulator of mitochondrial biogenesis and function. It interacts with nuclear receptors such as the peroxisome proliferator activated receptor- γ (PPAR γ), which in turn regulates several genes (29). PGC-1 α expression abnormalities have been associated with a number of neurological diseases like Huntington (30), Alzheimer (31) and Parkinson (32).

In brain tissue, PGC-1 α is particularly concentrated in inhibitory neurons and it is mandatory for the expression of the interneuron-specific calcium-binding protein parvalbumin in the cortex (33). Its role in the GABAergic system has remained unstudied.

The role of PGC-1 α was studied in immunoblotting and quantitative polymerase chain reaction (qPCR) experiments as a part of a larger study (34). Results suggest that the expression of GABA-α2 is enhanced in PGC-1 α transgenic mice in comparison to wildtype. In order to support these results, we stained brain sections from both PGC-1 α transgenic mice as well as wildtype, control mice and compared the expression of GABA-α2 in the CA1 hippocampal area.

1.2.6 Image differentiation using a deep learning CNN approach

To test if there was a difference in staining patterns of the GABA-α2 receptor, a machine learning technique was used in addition to a traditional image analysis. Specifically, Convolutional Neural Networks (CNN or Convnets), which is a subfield of machine learning, were used to differentiate images. Convnets have been widely used in the last couple of years as a tool for differentiating

between images and are becoming a popular diagnostic tool in radiology, including detection of many diseases such as pulmonary embolism, breast cancer, neurologic diseases and stained neuron detection (35-37). The underlying idea is that the basic unit of the input data comprises a 2D dimensional array which is then passed through a sequence of filters or kernels, extracting features from the image to make it classifiable (38).

Machine learning algorithms are applied to a given set of data or images (in our case, hippocampal cell images) and to some knowledge about these data (in this case, transgenic mice or wildtype). The data is broken down to smaller batches since the whole dataset can not be passed through the neural network at once. The algorithm system of convnets attempts to find right parameters for the filters that allow the separation between treated versus non-treated images. This process is known as a training process. Once the training step is finished, labeled images kept in the training process are presented to the algorithm (without the label) to test the degree of accuracy to which the algorithm is able to differentiate them (test step). In our case, the program made a prediction if the image is depicting a transgenic or wildtype mouse brain slice. At this point, it is possible to calculate the algorithm performance, e.g. 70 % of accuracy, which means that the algorithm correctly classifies 70% of the images presented. Afterwards, the algorithm can be further fine tuned to improve its accuracy based on medical knowledge. The algorithm system then optimizes its parameters such that its performance improves in that more test cases are diagnosed correctly (Fig. 5) (39).

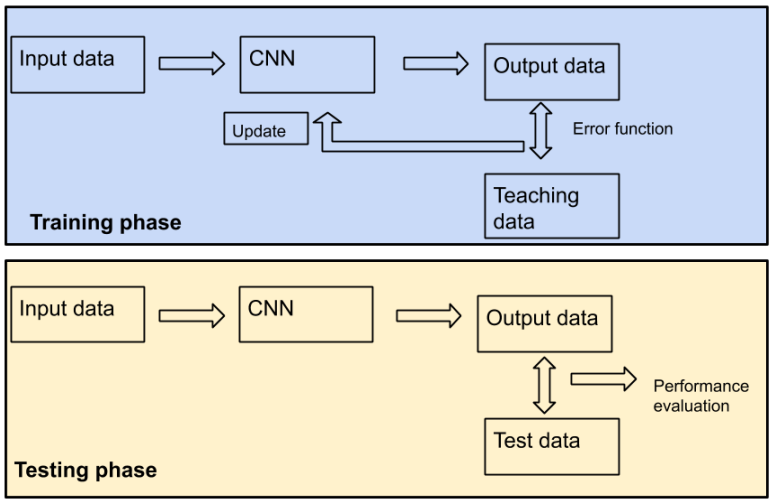


Figure 5. General principle of the CNN learning process. Redrawn from Yasaka, Akai et al. 2018 (36).

1.2.7 Rationale

In this study, we were interested in finding an optimal double staining protocol for GABA-α2 and CAMKII in mouse hippocampal pyramidal neurons in the CA1 region. This was done in order to visualize that GABA-α2 is expressed more frequently in hippocampal neurons in PGC-1α TG mice compared to wildtype mice (34).

Here I describe a journey on finding a working protocol for studying GABA-α2 expressing neurons in mouse hippocampal pyramidal cells. Currently, there is a large number of commercial antibodies sold for this purpose. A review of literature and current availability of antibodies decreased the number of tested antibodies to a few different brands. We stained our way through a number of different dilutions on both primary and secondary antibodies, with and without antigen retrieval in different temperatures and using both Triton-x and Tween-20 as detergents. Finally, we also tried two different thicknesses of sections.

Staining was done to test the null hypothesis that there is no difference in the expression of GABA-α2 receptors in the neurons of the hippocampal CA1 area in the two mouse groups (PGC-1α TG and wildtype mice).

Image analysis was performed using two different approaches; a traditional still image analysis of stained dot density and intensity and a deep learning CNN approach to study whether the staining patterns of GABAR-α2 in two categories of mice (transgenic/wildtype) can be distinguished using a computational algorithm.

2 MATERIAL AND METHODS

2.1 Handling of study animals

As a part of a larger study, PGC-1α TG mice with over-expression of PGC-1α under the Thy1 promoter in matured neurons were generated as described in Mudó (40) and backcrossed for several generations. C57Bl6/J mice were used as wildtype (WT) controls. Mice were anesthetized and perfused with 4 % paraformaldehyde in phosphate buffered saline after which the mice were decapitated and brains detached and post-fixed in 4% formaldehyde overnight at 4°C. The next day the brains were moved to 30% sucrose for cryoprotection overnight and then frozen and stored at -80 °C until analysis (around 4 months). Experiments were approved by the ethical committee and carried out in accordance with the European Communities Council Directive (86/609/EEC).

2.2 Staining tryouts

Brain sections including the hippocampal area of 20 µm were cut with a Leica CM3050S cryotome and stored in ethylene glycol in -20°C until staining (Fig. 4). Samples were then washed 3 x 5 minutes in phosphate buffered saline solution (PBS) prior to staining.

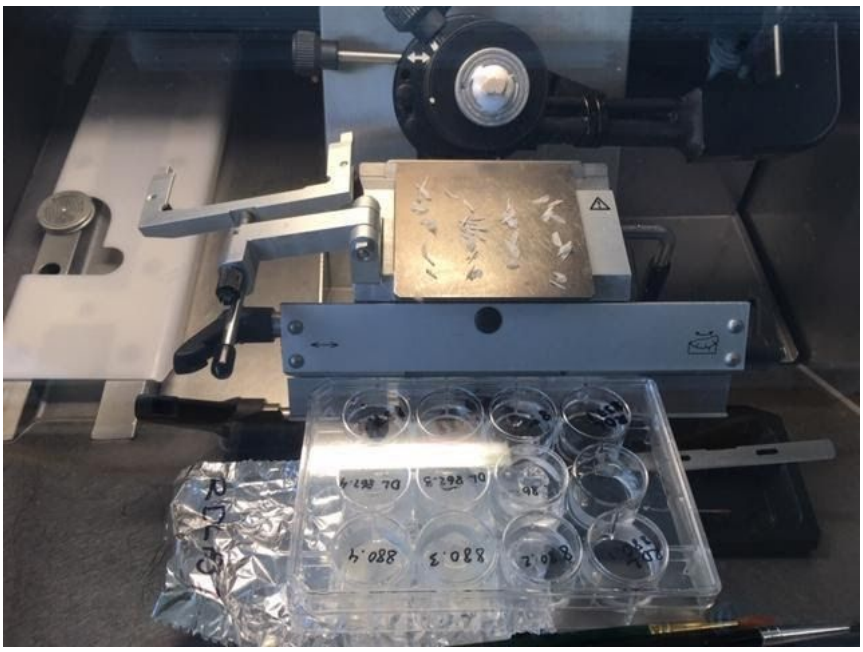


Figure 4. A Leica cryotome was used to cut brain tissue into desired thicknesses.

2.3 Staining of pyramidal cells with CAMKII

For the staining with CAMKII, two different brands and three different dilutions were tested. Staining was also performed using a HIER antigen retrieval in a 10 mM citrate buffer with three different temperatures (80°C, 85°C and 90°C) using a water bath incubation and without any antigen retrieval. The details for the tested CAMKII staining protocols are given in Appendix 1. Staining was tested on two different section thicknesses, 20 µm and 30 µm and using both Triton-x and Tween-20 as detergents. Before staining, blocking was performed using 1% BSA, 10 % serum of the secondary host species and either 0.2% triton or 0.2% tween-20 and phosphate saline buffer (pH 7.4) as a soluble for 1 h in room temperature. The primary antibody was added to the blocking solution and left for incubation for 24 h in 4°C. As negative controls, simultaneous stainings were done using similar protocols but lacking the primary antibodies (not included in the appendix). Secondary antibodies were added after 3 x 10 minutes washes in PBS and left for incubation 2h at room temperature. Sections were then mounted to microscopy slides using a thin brush and coverslipped using Vectashield with DAPI-stain.

2.4 Staining of GABA-α2

As for the CAMKII, several tryouts were stained with the anti-GABA-α2. Two different brands were tested, four different dilutions of primary antibody, two dilutions of secondary antibody, three different temperatures of HIER and two detergents (Triton-x and Tween-20). In addition, the protocol from Hines, Maric et al. 2018 (16) was followed using a modified blocking solution and a shorter incubation period for the secondary antibody. Full details of the tested protocols are summarized in Appendix 2.

2.5 Double staining

After finding a working staining protocol for both CAMKII and GABA-α2, a double staining was performed. Both primary antibodies were added simultaneously using a blocking solution with 1% BSA, 10 % secondary host species serum, 0.2% tween-20 with phosphate buffer as a soluble. The following protocol was used:

1st ABs	2nd AB + stain	AR	Block
GABA-α2 SySy 1:500 & CAMKII Sigma 1:500	donkey anti-rb AF488 1:700 & donkey anti-ms AF594 1:600	85°C 10 min in citrate buffer with 0.05% tween	1 % BSA, 10 % donkey serum, 0.2 % tween-20

2.6 Imaging of hippocampal cells and analytical tools

In order to test for differences in the GABAR-α2 units in the CA1 hippocampal area in TG mice compared to the control group, a single staining using anti-GABAR-α2 (rabbit polyclonal 1:500) as well as a double staining were performed. The multi-staining of GABAR-α2 and CAMKII was in order to distinguish if there is a co-localization of GABAR-α2 and pyramidal neurons. Prior to staining, samples were subjected to a heat-induced antigen retrieval in 10 mM citrate buffer including 0.05% tween-20 (85°C 20 min) and incubated in a blocking solution (1% BSA, 10 % donkey serum, 0.2% tween-20) for 1 h. After addition of the primary antibody, samples were incubated 24 h in 4°C. Alexa AF488 was used as a secondary antibody (donkey anti-rabbit 1:700) in blocking solution for 2 h at room temperature.

Images including z-stacks from the CA1 hippocampal area were taken using a LEICA SP8 confocal microscope. Three mice per genotype were used, including three brain sections, each photographed in four CA1 locations (a total of N = 36 per group). Images were analyzed with ImageJ using the ComDet v.0.4.1 plugin. Intensity and density of stained particles was calculated and compared using an independent samples t-test. Kolmogorov-Smirnov and Levene's tests were used to check that data fulfilled assumptions for parametric testing. All statistical testing was done using SPSS 25.

A second approach using deep learning was used to study whether the GABAR-α2 stained images differ between the two groups of mice. We used multichannel image stacks of our brain sections as data for a CNN image analysis, using 504 images in total per mouse brain type (transgenic vs. wildtype). Training and analysis were completed using Python 3.7. The model was implemented in the TensorFlow deep learning framework, which was run with GPU (8 G). We used 9 layers and tried different combinations of neurons and batches until a good model was found. This model included 512 neurons and 32 batches. Images were converted into grayscale pictures to be inputted into our model.

To evaluate the performance of our model, the results were quantitatively evaluated by 3 metrics, these being accuracy (ACC), sensitivity (SEN), and specificity (SPEC), and were defined as the following:

$$Sen = \frac{TP}{TP + FP}$$

$$Spec = \frac{TP}{FP + TN}$$

$$Acc = \frac{TP + TN}{TP + TN + FP + FN}$$

where TP (= true positive) is the number of true “positives” (transgenic mouse brain slice images) categorized correctly, TN (=true negative) is the number of “negatives” (wildtype mouse brain slice images) categorized correctly. FP (=false positive) is the number of wildtype mouse brain images misclassified as transgenic mouse images. FN (=false negative) is the number of transgenic mouse brain images misclassified as wildtype mouse brain images.

A ROC curve (receiver operating characteristic curve) and a precision-recall curve was then used as diagnostic tools to help in the interpretation of binary classification predictive models and to clarify the trade-off in sensitivity and precision.

3 RESULTS

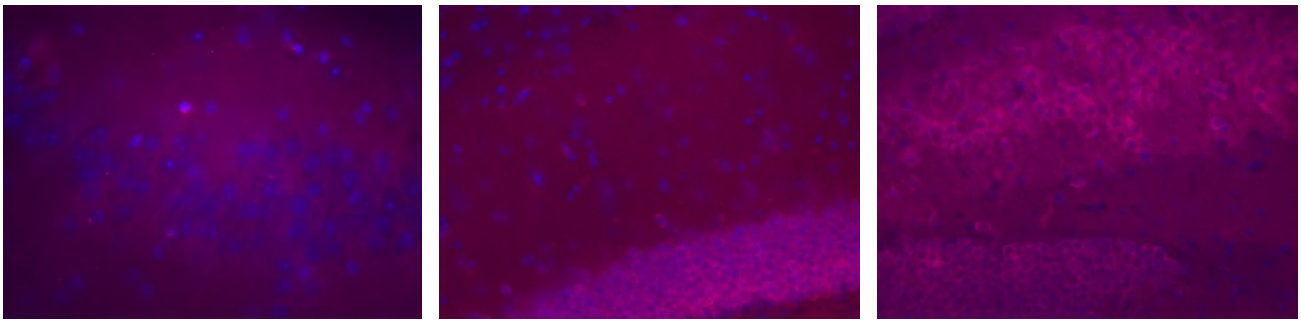
3.1 CAMKII staining of pyramidal cells

In the staining of CAMKII, a HIER was needed to visualize the pyramidal cells. After antigen retrieval, both primary antibody dilutions worked well (1:250, 1:700). The manufacturer recommended a dilution of 1:100. Both brands stained clearly after the HIER. As a detergent, Tween-20 seemed to be more compatible although Triton also worked (Fig. 6). Despite clear staining, a high amount of background noise appeared in all trials.

A. CAMKII SigmaAldrich 1:700, magnification x 63, no AR, triton

B. CAMKII SigmaAldrich 1:700, magnification x 40
AR 80°C 20 min, triton

C. CAMKII SigmaAldrich 1:700, magnification x 40
AR 85°C 25 min, triton



D. CAMKII Abcam mouse 1:250, magnification x 63
AR 80°C 20 min, triton

E. CAMKII Sigma mouse 1:250, magnification x 40
AR 80°C 20 min, tween-20

F. CAMKII Abcam mouse 1:250, magnification x 40
AR 80°C 20 min, tween-20

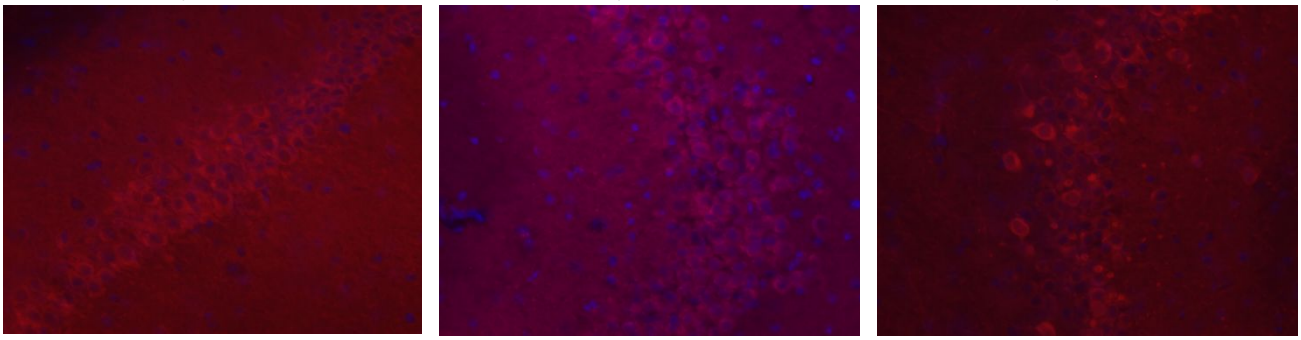


Figure 6. Staining outcomes for CAMKII positive pyramidal neurons. Without antigen retrieval, no pyramidal cells were detected (A), whereas both antibodies and used dilutions stained well after antigen retrieval (B-F; red/pink cells). Pictures taken with a Leica Axioplan 2 imaging microscope. AR = antigen retrieval

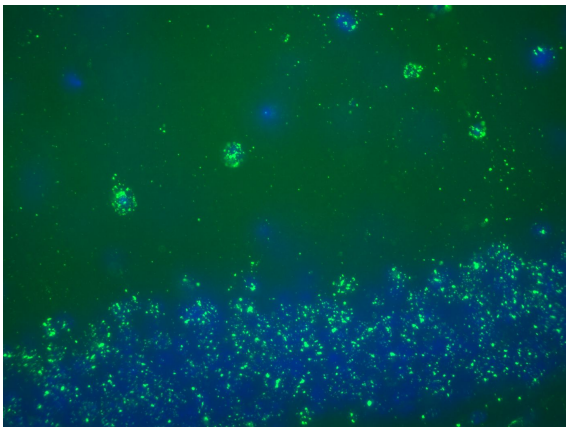
3.2 Staining of GABA-α2

In the staining of GABA-α2, there was a clear difference between the two brands tested. The antibody from SantaCruz resulted in diffuse, intense dot-like staining, which was also found in the negative control and therefore deemed as unspecific staining. Using the antibody from Synaptic

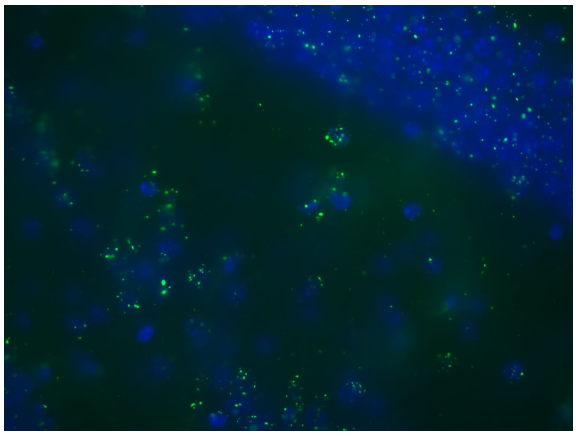
Systems, the whole cell soma became stained in a more diffuse pattern and no resembling patterns were found in the negative controls (Fig. 7).

The protein of interest was barely detected using triton as a detergent. Like CAMKII, the antibody needed a HIER in citrate buffer to become visible. In all samples, there was a high amount of background staining. The protocol from Hines et al. (16) did not stain in our trials.

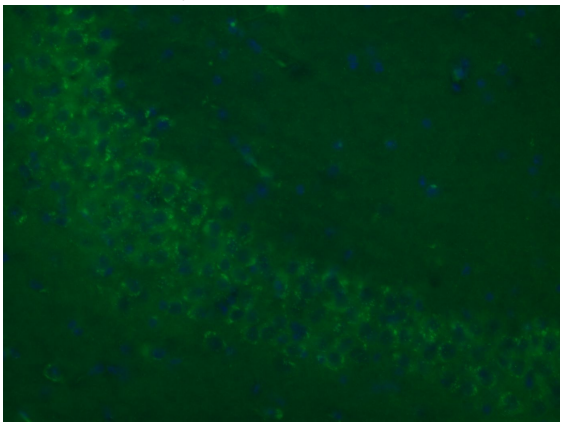
A. GABA SantaCruz 1:500, magnification x 40
AR 85°C 25 min, triton



B. GABA SantaCruz 1:1000, magnification x 40
AR 85°C 25 min, triton



C. GABA SySy 1:500, magnification x 40
AR 80°C 25 min, tween-20



D. GABA SySy 1:500, magnification x 63
AR 80°C 25 min, tween-20

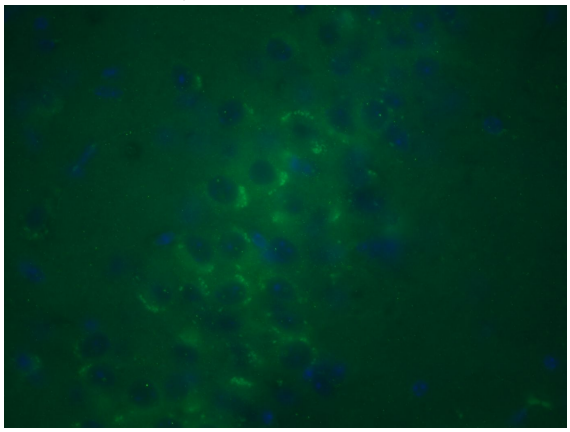


Figure 7. The SantaCruz antibody resulted in dot-like staining (A-B) and similar staining was also detected in negative control samples. Using tween-20 as a detergent, GABA-α2 staining was detectable. Pictures taken with a Leica Axioplan 2 imaging microscope. AR = antigen retrieval

3.3 Double staining

The double staining resulted in clearly detectable pyramidal cells in the hippocampal area when subjected to HIER and using Tween-20 as detergent (Fig. 8). Imaging was done using a LEICA SP8 confocal microscope. A section thickness of 20 µm was used for the actual samples when comparing the TG mice to the controls since it was deemed easier to image. Also, sections were mounted without the DAPI-stain since our tryout sections all had a significant amount of background noise. Despite this, the amount of background noise in samples lacking DAPI-stain remained high.

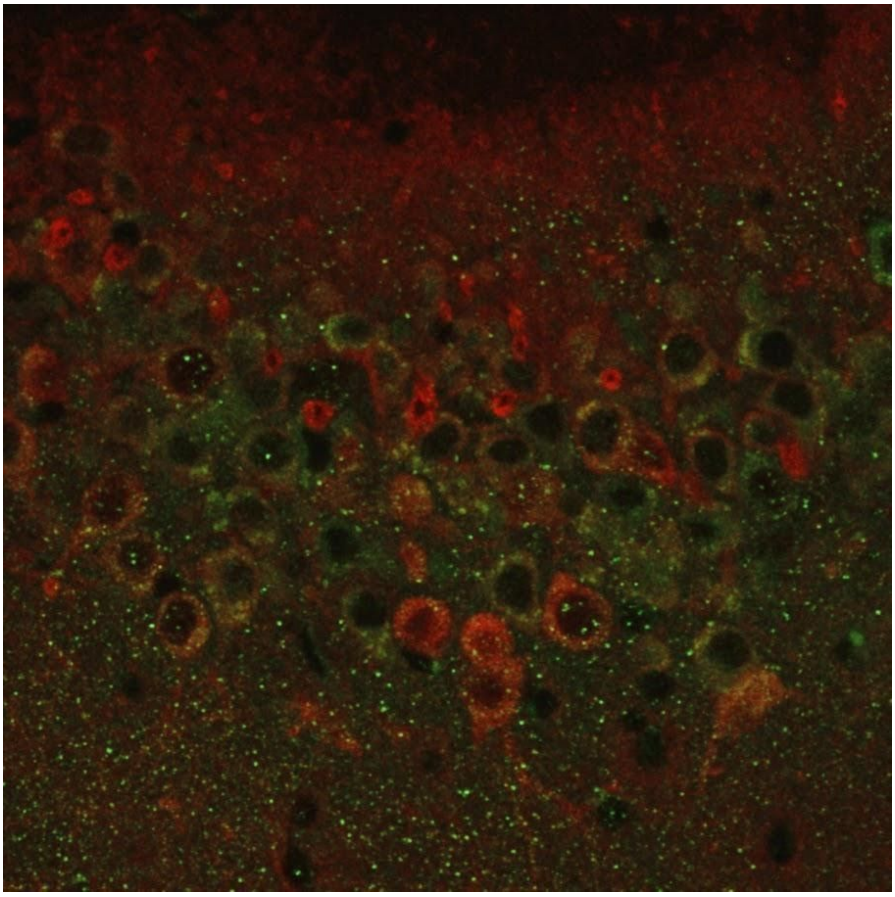


Figure 8. Pyramidal neurons stained in red AF594 using anti-CAMKII and GABA- α2 in green AF488 stain. Picture taken using a LEICA-confocal microscope, magnification x 63.

3.4 Staining of hippocampal cells

No differences in the staining intensity or density between the two study groups were detected when analyzing the still images in the ImageJ program (Fig. 9).

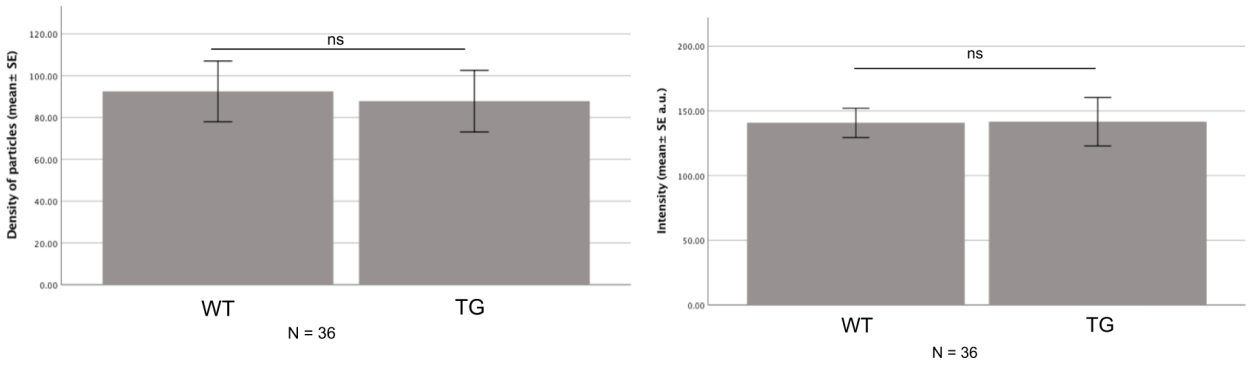


Figure 9. Density and intensity of stained particles in still pictures showed no differences between the two groups of mice. WT = wiltype mice, TG = PGC-1 α transgenic mice

Using the deep learning approach, the algorithm was able to separate the two image groups with an accuracy of 100% during training process. Nevertheless, when the model was evaluated on the test data, the accuracy went down to 72% using a decision boundary probability of 0.85 (Table 1). A decision boundary probability of 0.5 is used by a trained machine learning algorithm to separate two treatments. It is possible to increment this probability to increment the accuracy, being more or less restrictive. In this case, an increment of the decision boundary probability incremented the accuracy in 2%, resulting in a 72% overall accuracy (total % images categorized correctly).

Table 1. Classification report. 1 and 0 refer to the test groups (TG/wildtype mice).

	precision	recall	f1-score	support
1	0.67	0.88	0.76	50
0	0.83	0.57	0.67	51
accuracy			0.72	101
macro avg	0.75	0.72	0.72	101
weighted avg	0.75	0.72	0.72	101

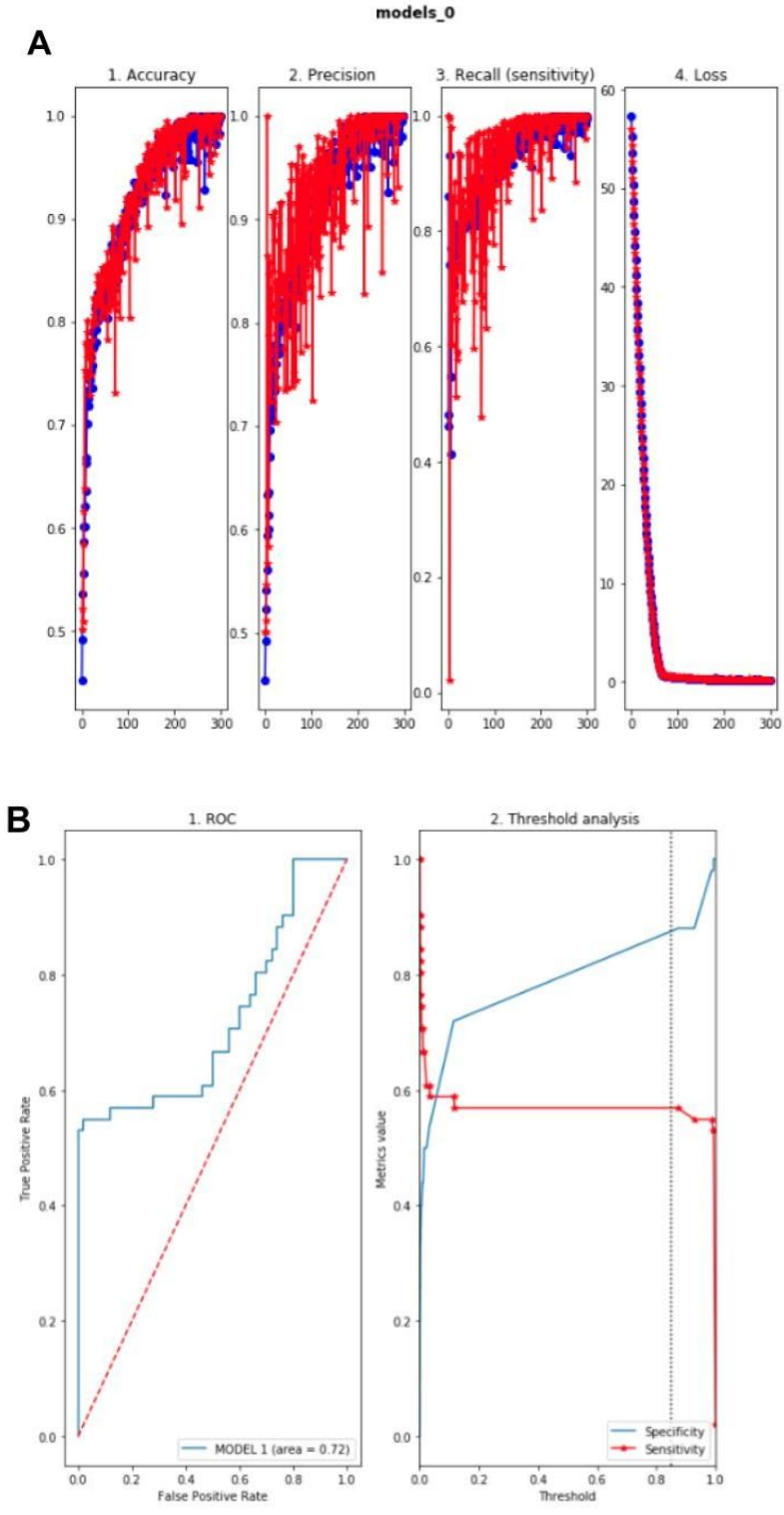


Figure 10 a-b. A total of 300 batches were run, during which the accuracy of the model rose from 45% to 100%. Likewise, the precision (= specificity) and sensitivity (=recall) in the model showed a sharp rise after training. Loss refers to the error at the output layer and decreased during training. The area under the ROC curve constituted 72%. The threshold precision-recall curve, “Threshold analysis”, shows that in our model with the highest accuracy, there is a relatively high specificity (83%) while the sensitivity drops to 58%.

The ROC curve is a graph showing the performance of a classification model at all classification thresholds. The area under the ROC curve was 72%, in accordance with the accuracy of the model. The total sensitivity and specificity of our model were 82.8 % and 58.0 %, respectively when the threshold was set to 85 % (Fig 10b).

4 DISCUSSION

4.1 Challenges of double staining

Although protocols for antibody staining are often provided by the antibody manufacturer, the information can be regarded only as a loose guideline and researchers always need to adapt the antibody protocol to the prevailing laboratory conditions. The situation becomes even more complicated when performing a double-stain since a working protocol for one of the antibodies may not be compatible with the other; the antibodies may cross-react, have a high background noise for various reasons, need different detergents and be visualized so that both antigens are detectable. In addition, the staining procedure is different if the primary antibodies are raised in the same or in different species and if the antigens of interest are located in the same or in different cell compartments (e.g cytosol, dendrites). Trying out different versions may soon become very time and money consuming. Since the approach is always based on avoiding problems, the double staining is rarely optimal for both proteins of interest.

In our case, the double staining was relatively straightforward after finding a working HIER, which turned out to be the crucial step in this staining. Double staining is limited by the fact that it may not be possible to find a single tissue pre-treatment protocol that is optimal for all targets. Hence, it may be necessary to find a compromising method that allows all targets to be stained. In our case, the pre-treatment was an HIER in 85°C water bath for 10 minutes in a 10 mM citrate buffer using 0.05% tween as a detergent.

4.2 Staining of CAMKII and GABA-α2

The staining of CAMKII was used to mark pyramidal and other excitatory cortical neurons excluding GABAergic interneurons. This is a commonly used antibody (15) and stained well after the HIER using a number of brands and dilutions.

With the staining of GABA-α2, we did not manage to get a clear staining in comparison to the negative control samples lacking the primary antibodies in several of the tryouts. The background noise also remained high throughout the tryouts. The staining was especially challenging considering the very different staining patterns using the different brands – “dots” versus staining of somal structures (Fig. 7) and considering that both patterns have been used as examples in peer

reviewed publications – somal structures (16); "dots" (41). Staining patterns are also likely to differ depending on scale and microscope type. Using triton as detergent, only weak signaling could be detected. One reason for this might be that Triton-x is generally considered a harsh detergent (13), possibly disrupting the cell membrane and leading to the loss of surface proteins.

The discrepancy in GABA-α2 staining between the different brands was obvious and remained a mystery. No reviews regarding the Santa Cruz Biotechnology antibody was found online, however, according to the manufacturer, the SC-7350 antibody is no longer being sold.

Another factor that can have affected the staining outcome of GABA-α2 was that the antibody we used was polyclonal. Polyclonal antibodies are typically raised in rabbits using traditional immunization techniques. This tends to give them a higher sensitivity (avidity) compared to monoclonal antibodies since they contain a higher number of antibody species able to react with the antigen sites. Polyclonal antibodies have an advantage in being able to escape harmful effects of tissue fixation and processing. However, more antigen sites are recognized by the polyclonal antibodies and the risk of cross-reaction to other proteins is increased and may result in "dirty" staining (3).

4.3 Handling and preparation of samples

In this study, a whole animal perfusion technique was used with formaldehyde fixation. This technique can mask epitopes and limit antibody-epitope binding. Of the many pre-analytical steps prior to staining, fixation is probably the most crucial step affecting a number of other variables (3). The staining protocols in published literature usually refer to either paraffin embedded or fresh frozen samples. Also, most protocols recommend cutting the brain immediately after perfusion (8), which was in contrast to the handling procedure in this experiment.

Antigen retrieval is known to be required for successful staining when using paraffin-embedded tissue sections whereas less is known for frozen paraformaldehyde pre-fixed samples (42). Our tryouts clearly showed that a HIER was needed for detecting desired antigens although it also compromised the morphology of the sections, making them wrinkly in many cases. A temperature of 80°C was sufficient to reveal our antigens of interest although temperatures exceeding 90°C are usually recommended (42).

4.4 PGC-1 α transgenic mice

In previous studies, GABA-α2 has been localized especially to synaptic regions in the axon initial segment where action potentials initiate and in clusters along axons (43). This can partly explain why no differences between staining patterns were detected between mouse groups using the still images with ImageJ since these pictures contained mostly cell soma. When the multistack images containing also the dendrites were analyzed using the CNN approach, a clear imbalance was detected between the study groups. An increased expression of GABARα2 in the PGC-1α TG could affect the behaviour and decision making of mice, such as spatial learning and anxiety.

4.5 Image analysis

In recent years, deep learning has become one of the most popular methods in computational image analysis. CNNs with self-learning abilities are of high use in medical image classification, segmentation and detection. It has great advantages compared to traditional methods and is bringing about a shift in paradigm in healthcare (38). Until now, CNN analytical tools have mostly been used for diagnosing purposes, but it has also been used to successfully detect mutated histopathological images (44) and to classify and segment microscopy images (45).

The high accuracy and specificity of our model shows promising results for detecting differences in IHC stained images that traditional image analysis tools are unable to detect. This could be of high importance in future IHC work and I believe that the model structure used in this study could be applied on other similar imaging approaches. That said, the CNN image analysis approach has a few challenges.

The learning ability of CNNs function via error backpropagation and stochastic optimization to categorize the images to their respective classes. The models are highly sensitive to the training data fluctuations, which can result in modeling random noise and overfitting during training. This sometimes leads to high prediction variance and limited performance (46). Deep learning is very demanding in terms of training data which needs to be extensive. Using our image data, this shortcoming was compensated for by the incorporation of data augmentation (rotation, resizing of images).

4.6 Conclusions

Staining can be very time-consuming and expensive considering the amount of antibodies on the market. Compromises regarding pre-treatment, cell membrane penetration and visualization have to be made in order to get a working protocol for both antigens. Having a pretested protocol for multi-staining can be very cost-effective if one wishes to conduct further research with the same proteins. Having found a working protocol, the next step is to conduct image analysis of the stained samples. In this study, we tested a novel CNN deep learning approach and conclude that the CNN model worked well for categorization of our samples, superior to traditional image analysis approaches.

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Appendix 1

1st AB	2nd AB + stain	AR	Block	Detergent
1:700 CAMKII mouse monoclonal Sigma Aldrich C265	Goat anti-mouse 1:700 + AF647	-	1 % BSA 10 % goat serum 0.3% triton	Triton-x
1:700 CAMKII mouse monoclonal Sigma Aldrich C265	Goat anti-mouse 1:700 + AF647	80°C 20 min	1 % BSA 10 % goat serum 0.3% triton	Triton-x
1:250 CAMKII mouse monoclonal Sigma Aldrich C265	1:700 goat anti-mouse + AF 647	80C 20 min	1 % BSA 10 % goat serum 0.3% triton	Triton-x
1:250 CAMKII mouse monoclonal Sigma Aldrich C265	1:700 goat anti-mouse + AF 647	85C 25 min	1 % BSA 10 % goat serum 0.3% triton	Triton-x
1:250 CAMKII mouse monoclonal Sigma Aldrich C265	1:700 goat anti-mouse + AF 647	85C 25 min	1 % BSA 10 % goat serum 0.3% triton	Triton-x
1:250 CAMKII mouse monoclonal Sigma Aldrich C265	1:600 anti-mouse goat + AF 647	93 C 25 min	1 % BSA 10 % goat serum 0.2% triton	Triton-x
1:500 CAMKII mouse monoclonal Sigma Aldrich C265	1:600 donkey anti-mouse AF594	85°C 20 min	10 % donkey, 1 % BSA, 0.2 % tween	Tween-20
1:250 CAMKII Abcam mouse monoclonal	1:600 donkey anti-mouse + AF594	85°C 20 min	10% donkey, 1 % BSA, 0.2 % tween	Tween-20
1:250 CAMKII Abcam mouse monoclonal	1:600 donkey anti-mouse + AF594	85°C 20 min	10 % donkey, 1 % BSA, 0.2 % triton	Triton-x

Appendix 2

1st AB	2nd AB + stain	AR	Block	Detergent
1:1000 GABRA2, Santa Cruz goat polyclonal SC-7350	1:700 donkey anti-goat, AF 488	85C 25 min	1% BSA 10 % donkey serum 0.2% triton	Triton-x
1:500 GABRA2, Santa Cruz goat polyclonal SC-7350	1:700 donkey anti-goat, AF 488	85C 25 min	1% BSA 10 % donkey serum 0.2% triton	Triton-x
1:1000 GABRA2, Santa Cruz goat polyclonal SC-7350	1:700 donkey anti-goat, AF 488	80C 20 min	1% BSA 10 % donkey serum 0.2% triton	Triton-x
1:500 GABRA2, Santa Cruz goat polyclonal SC-7350	1:700 donkey anti-goat, AF 488	80C 20 min	1% BSA 10 % donkey serum 0.2% triton	Triton-x
1:500 GABA-α2 goat Santa Cruz	1:700 anti-goat donkey AF 488	93 C 25 min	1% BSA 10 % donkey serum 0.2% triton	Triton-x
1:500 GABA-α2 goat Santa Cruz	1:600 anti-goat donkey AF 647	93 C 25 min	1% BSA 10 % donkey serum 0.2% triton	Triton-x
1:1000 GABA-α2 goat Santa Cruz	1:700 anti-goat donkey AF 488	93 C 25 min	1% BSA 10 % donkey serum 0.2% triton	Triton-x
1:700 GABRA2 rabbit polyclonal 224103 SySy	1:700 donkey anti-rabbit, AF 488	85C 25 min	1% BSA 10 % donkey serum 0.2% triton	Triton-x
1:500 GABRA2 rabbit polyclonal 224103 SySy	1:700 donkey anti-rabbit, AF 488	85C 25 min	1% BSA 10 % donkey serum 0.2% triton	Triton-x
1:1000 GABA-α2, rabbit polyclonal Synaptic System 224103	1:700 goat anti-rabbit + AF 594	-	1% BSA 10 % goat serum 0.3% triton	Triton-x
1:700 GABA-α2, rabbit 1:1000 Synaptic System 224103		-	1% BSA 10 % goat serum 0.3% triton	Triton-x
1:600 GABRA2 rabbit Synaptic Systems *	1:700 anti-rabbit donkey AF 488	93 C 25 min	1% BSA 10 % donkey serum 0.2% triton	Triton-x

1:500 GABRA2 SySy rabbit polyclonal 224103	1:500 anti-rabbit donkey AF 488	85°C 25 min	1% BSA 10 % donkey serum 0.2% triton	Triton-x
1:500 GABRA2 SySy rabbit polyclonal 224103	1:700 anti-rabbit donkey AF 488	Citrate buffer with Tween 0.05%, 85°C 25 min	1% BSA 10 % donkey 0.2% tween	Tween-20
1:500 GABRA2 SySy 224103 with protocol from Hines et al. 2018	1:700 anti-rabbit donkey AF 488	-	2.5% BSA, 5% donkey serum, 0.1% triton-x. Incubations in modified block with 2 % donkey serum.	Triton-x